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# In Vitro Studies of Tobramycin, an Aminoglycoside Antibiotic

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Tobramycin is an aminoglycoside antibiotic which has excellent antibacterial activity against *Pseudomonas, Staphylococcus aureus*, and many members of the *Enterobacteriaceae*. Most strains of *Serratia, Providence, Streptococcus*, and *Diplococcus pneumoniae* were resistant to concentrations of tobramycin which could be achieved in man. Tobramycin was effective against certain *Pseudomonas* strains resistant to gentamicin. The growth medium used to determine the inhibitory level of tobramycin had a significant effect upon the minimal inhibitory concentration. Calcium and magnesium ions inhibited the bactericidal effect of tobramycin. Tobramycin and carbenicillin acted in a synergistic manner. Ethylenediaminetetraacetic acid did not act in a synergistic manner with tobramycin. Broth-dilution susceptibility tests and disc-diffusion tests in agar (10-$\mu$g discs) showed excellent correlation except with *Proteus* strains.

Tobramycin is a new aminoglycoside antibiotic derived from nebramycin, an antibiotic complex produced by *Streptomyces tenebrarius* (4). It has been reported to have antibacterial activity against most of the *Enterobacteriaceae*, *Pseudomonas* species, and *Staphylococcus aureus* (3, 6, 9). The increasing importance of gram-negative infections and the appearance of certain bacteria resistant to gentamicin (7) prompted us to study the antimicrobial activity of tobramycin against isolates from an institution in which there has been extensive use of kanamycin and gentamicin. The evaluation of tobramycin in such a setting gives a more realistic view of the potential of the agent.

## MATERIALS AND METHODS

Tobramycin (nebramycin factor 6) was supplied as a sterile liquid by Eli Lilly & Co. Fresh dilutions were prepared daily in sterile medium or buffer. Carbenicillin was a gift of Beecham Pharmaceuticals.

The bacteria tested were isolated from patients hospitalized at the Columbia-Presbyterian Medical Center.

**Susceptibility-testing methods.** The activity of tobramycin was measured by the broth-dilution method. Serial twofold dilutions in Brain Heart Infusion (Difco) were used with 0.5 ml of inoculum from an overnight culture containing $10^4$ cells. Final volume was 1 ml. Incubation was for 18 hr at 35 C. The minimal inhibitory concentration (MIC) of the antibiotic was defined as the lowest concentration which inhibited development of visible turbidity. The minimal bactericidal concentration (MBC) was determined by plating clear tubes from the broth-dilution series. MIC values were also determined by the agar-dilution method. An overnight culture was used, and the organisms were applied with a replicating device.

Studies of the synergy of tobramycin with carbenicillin and ethylenediaminetetraacetic acid (EDTA) were performed by use of twofold serial dilutions in a checkerboard tube system previously described (5).

Disc susceptibility tests were performed by the method of Bauer et al. (1) with the use of 6-mm paper discs containing 10 $\mu$g of tobramycin. The susceptibility of the test organisms to other antibiotics was also determined by the Bauer-Kirby (1) method. For these tests, commercial discs containing the following amounts of each drug were used: gentamicin, 10 $\mu$g; kanamycin, 30 $\mu$g; cephalothin, 30 $\mu$g; ampicillin, 10 $\mu$g; colistin, 10 $\mu$g; chloramphenicol, 30 $\mu$g; and tetracycline, 30 $\mu$g.

The basal medium used contained 100 mM tris(hydroxymethyl)aminomethane-hydrochloride 15 mM $NH_4Cl$, 0.625 mM $Na_2SO_4$, 1 mM KCl, 0.62 mM sodium phosphate, and 0.2% glucose. The concentrations of $MgCl_2$ and $CaCl_2$ were varied as noted from 0.1 to 2.5 mM.

The effect of variation in media was determined with Trypticase Soy (BBL), nutrient (Difco), Mueller-Hinton (BBL), and Brain Heart Infusion (Difco) broths.

## RESULTS

Tobramycin has a wide spectrum of activity against gram-negative organisms in vitro. Figure

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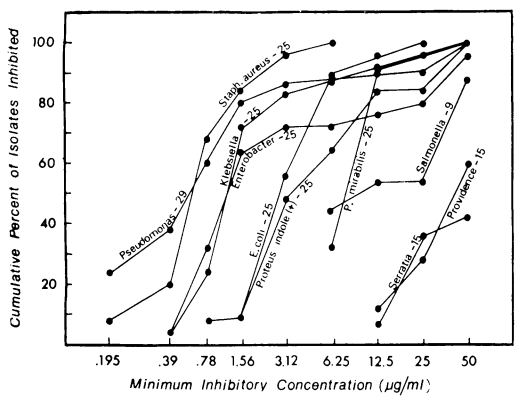


FIG. 1. *Susceptibility of various clinical isolates to tobramycin. The number of strains tested is indicated.*

1 shows the cumulative MIC of tobramycin against *Pseudomonas*, *Enterobacter*, *Klebsiella*, *Escherichia coli*, *Proteus mirabilis*, indole-positive *Proteus*, *Serratia*, *Providence*, *Salmonella*, and *Staphylococcus aureus* strains. Of the *Pseudomonas aeruginosa* strains tested, 90% were inhibited by 6.25 μg/ml and 80% by 1.56 μg/ml. A concentration of 6.25 μg/ml inhibited 92% of *E. coli* strains, 72% of *Enterobacter* species (*E. cloacae*, *E. aerogenes*, and *E. hafnia*), 88% of *Klebsiella* strains, and 64% of indole-positive *Proteus* strains (*P. morganii*, *P. rettgeri*, and *P. vulgaris*). Among the 25 *S. aureus* strains, 85% were susceptible to 1.56 μg/ml and 100% were susceptible to 6.25 μg/ml. The 25 *S. aureus* strains included 5 methicillin-resistant strains and 3 kanamycin-resistant strains.

*P. mirabilis* strains were less susceptible to tobramycin; only 32% were inhibited by 6.25 μg/ml. *Serratia marcescens* and *Providence* were generally resistant to more than 25 μg of tobramycin/ml. The MIC values of tobramycin against the few *Salmonella* strains tested were relatively high.

Table 1 shows the comparative susceptibility of several microorganisms to antibiotics currently used in this institution. Gentamicin, in spite of extensive use at this institution, remains the most active agent against most gram-negative bacteria. Tobramycin had a spectrum of activity comparable to that of gentamicin except against *Serratia*, *Providence*, and *Proteus*. The activity spectrum of tobramycin was quite similar to that of kanamycin except for its excellent activity against *Pseudomonas aeruginosa* and relatively poor activity against *Proteus* strains.

Tobramycin showed poor activity against five isolates of *Streptococcus pyogenes*, five isolates of *S. faecalis*, and five of *D. pneumoniae*. All were resistant to 12.5 μg/ml.

In paired broth-dilution tests of tobramycin and gentamicin against 13 *Pseudomonas* strains, tobramycin was consistently two- to fourfold more active (Table 2).

**Effect of medium and size of inoculum.** In general, the size of inoculum had little effect upon the MIC of tobramycin (Table 3). However, the MIC for some strains became much larger when the inoculum was increased from $10^5$ to $10^7$ colony-forming units (CFU). This is illustrated by *S. aureus* 850, against which the MIC was 50 μg/ml with an inoculum of $10^7$ CFU but 0.19 μg/ml with $10^5$ CFU. The MBC was not influenced by the inoculum size. Tobramycin was active against some strains which were resistant to gentamicin, and in the matched series was consistently two- to fourfold more active than gentamicin. The two strains resistant to tobramycin were also resistant to gentamicin.

The effect that the medium used in an assay had upon the MIC and MBC may be noted in Table 4. A comparison of the results with nutrient broth (*p*H 6.8) and Mueller-Hinton broth (*p*H 7.4) suggests that the *p*H of the medium probably plays a significant role. Tobramycin was more active at the alkaline *p*H. However, differences in the calcium and magnesium content of the media undoubtedly affected the results also. Addition of heat-inactivated serum did not increase the MIC or MBC for any of the organisms tested. The MBC was rarely more than one tube greater (twofold) than the MIC for any of the organisms studied.

Comparison of the MIC obtained by serial broth dilutions and by an agar plate dilution method in which undiluted overnight cultures were used gave values which varied from twofold less to twofold greater without a consistent pattern. These two methods could not be readily compared, probably owing to the variable binding of cations by the agar.

The effect of cations, $Ca^{++}$ and $Mg^{++}$, was investigated by use of a minimal medium. The MIC was significantly lower in minimal medium at the same *p*H than in any of the enriched media; i.e., in Brain Heart Infusion, the MIC against *E. coli* was 12.5 μg/ml, that against *Klebsiella* was 25 μg/ml, and that against *Pseudomonas* was 50 μg/ml, whereas the respective MIC values in minimal medium were 1.56, 0.2, and 0.2 μg/ml. Increasing the concentration of $Mg^{++}$ from 0.1 to 2.5 mM and adding $Ca^{++}$ to 2 mM increased the MIC two- to eightfold. The studies suggest that $Ca^{++}$ is more effective in inhibiting tobramycin than $Mg^{++}$ at equimolar concentrations. In addition, regrowth of a

TABLE 1. *Comparison of tobramycin activity with the antibacterial activity of antibiotics currently in use*

| Organism | No. of strains tested | Percentage of strains susceptible[a] | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | Tobramycin | Gentamicin | Kanamycin | Cephalothin | Ampicillin | Colistin | Chloramphenicol | Tetracycline |
| *Staphylococcus aureus* | 25 | 100 | 100 | 84 | 100 | 23 | 0 | 92 | 55 |
| *Pseudomonas* | 29 | 90 | 85 | 0 | 0 | 0 | 100 | 4 | 0 |
| *Escherichia coli* | 25 | 92 | 100 | 100 | 78 | 77 | 96 | 92 | 58 |
| *Klebsiella* | 25 | 88 | 100 | 70 | 70 | 0 | 83 | 80 | 56 |
| *Enterobacter* | 25 | 72 | 100 | 87 | 10 | 13 | 84 | 88 | 70 |
| *Proteus mirabilis* | 25 | 32 | 100 | 95 | 100 | 100 | 0 | 88 | 0 |
| Indole-positive *Proteus* | 25 | 63 | 100 | 87 | 16 | 17 | 0 | 75 | 45 |
| *Serratia* | 15 | 0 | 100 | 35 | 5 | 10 | 23 | 72 | 12 |
| *Providence* | 15 | 0 | 66 | 75 | 20 | 33 | 0 | 22 | 0 |

[a] For tobramycin, susceptibility to 6.25 μg/ml was determined by the broth-dilution method. For the other drugs, susceptibility was determined by zone sizes by use of the Bauer-Kirby (1) technique.

tobramycin-resistant *Pseudomonas* strain in the presence of tobramycin and either $Mg^{++}$ or $Ca^{++}$ occurred first in the minimal medium containing $Ca^{++}$.

Rapidity of killing is shown (Fig. 2) for an *E. coli* and a *P. aeruginosa* strain. In 90 min, there was a two-log fall in colony counts. Strains which were resistant (Fig. 3) showed an initial killing effect, but those organisms which persisted eventually grew out. This was not due to destruction of the tobramycin. Agar plate dilutions of such strains showed a "skipped-plate" phenomenon with a few colonies present at high concentrations of the antibiotic.

The resistance of *Serratia* and *Providence* strains did not seem to be due to destruction of the compound, because the broth contained tobramycin at the original concentration when the strains were fully grown.

**Synergy studies.** The reports of synergy between the aminoglycoside antibiotics gentamicin and carbenicillin (8) prompted us to see whether tobramycin and carbenicillin also acted in a synergistic manner. Synergy between tobramycin and carbenicillin was studied with strains for which the MIC of tobramycin ranged from 50 to 3.12 μg/ml. An isobol plot of the MIC values for four *Pseudomonas* strains (three resistant and one susceptible) which were tested in a checkerboard broth-dilution test is shown in Fig. 4. The MIC of both tobramycin and carbenicillin was significantly lowered. In the presence of 25 μg of carbenicillin/ml, the average MIC of tobramycin was 0.19 μg/ml.

Similar synergy studies were performed for tobramycin and EDTA. The concentration of EDTA was varied from 1 mM to 6.25 μM. The effect was additive, and synergy was not shown for the *Pseudomonas*, *Enterobacter*, *E. coli*, and

TABLE 2. *Comparative susceptibility of 13 Pseudomonas strains to tobramycin and gentamicin*[a]

| Strain | Minimal inhibitory concn (μg/ml) | |
|---|---|---|
| | Tobramycin | Gentamicin |
| 916 | 1.56 | 50.0 |
| 1114 | 3.12 | 50.0 |
| 865 | 6.25 | 25.0 |
| 703 | 0.78 | 12.5 |
| 882 | 1.56 | 25.0 |
| 866 | 0.19 | 0.78 |
| 906 | 0.19 | 0.78 |
| 920 | 0.19 | 0.39 |
| 974 | 0.19 | 1.56 |
| 975 | 0.19 | 0.39 |
| 976 | 0.19 | 0.39 |
| 950 | 50.0 | 12.5 |
| 954 | 50.0 | 50.0 |

[a] Broth dilutions were performed in Brain Heart Infusion with an inoculum of $10^4$ organisms from an overnight culture.

TABLE 3. *Effect of inoculum size on susceptibility to tobramycin*[a]

| Strain | Minimal inhibitory concn (μg/ml) | | |
|---|---|---|---|
| | $10^7$ CFU[b] | $10^5$ CFU | $10^3$ CFU |
| *Pseudomonas* 919 | 0.39 | 0.19 | 0.19 |
| *Pseudomonas* 954 | >50 | >50 | >50 |
| *Staphylococcus aureus* 952 | 0.19 | 0.19 | 0.19 |
| *S. aureus* 850 | 50.0 | 0.19 | 0.19 |
| *Escherichia coli* 740 | 6.25 | 1.56 | 1.56 |
| *Proteus vulgaris* 962 | 12.5 | 1.56 | 1.56 |
| *Serratia* 955 | >50 | >50 | >50 |

[a] Brain Heart Infusion (BBL) was used.
[b] Inoculum (colony-forming units).

TABLE 4. *Effect of growth medium on susceptibility to tobramycin*[a]

| Organism | Medium | | | | |
|---|---|---|---|---|---|
| | BHI | BHI + serum | TSB | NB | MHB |
| *Staphylococcus aureus* | 0.19 (0.39) | 0.19 (0.39) | 0.78 (3.12) | Not done | 0.19 (0.19) |
| *Pseudomonas aeruginosa* | 0.19 (1.56) | 0.19 (0.19) | 0.19 (1.56) | 1.56 (1.56) | 3.12 (3.12) |
| *Proteus mirabilis* | 6.25 (50) | 1.56 (3.12) | 3.12 (12.5) | 6.25 (12.5) | 1.56 (6.25) |
| *Klebsiella pneumoniae* | 1.56 (3.12) | 1.56 (1.56) | 1.56 (6.25) | 3.12 (3.12) | 1.56 (1.56) |
| *Enterobacter cloacae* | 1.56 (1.56) | 0.19 (0.39) | 0.78 (3.12) | 6.25 (6.25) | 1.56 (1.56) |
| *Escherichia coli* | 3.12 (6.25) | 0.78 (0.78) | 25 (25) | 12.5 (12.5) | 12.5 (12.5) |

[a] Results show the minimal inhibitory concentrations with the minimal bactericidal concentrations given in parentheses (both are expressed in micrograms per milliliter). BHI, Brain Heart Infusion; TSB, Trypticase Soy Broth; NB, nutrient broth; MHB, Mueller-Hinton broth.

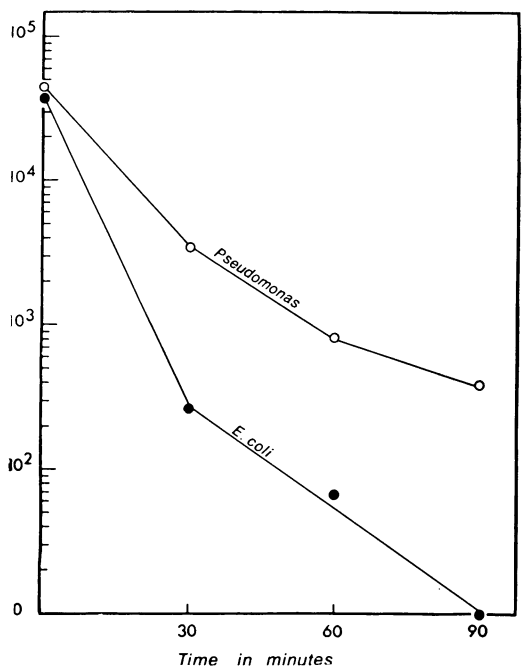


FIG. 2. *Bactericidal effect of tobramycin. Tobramycin (50 μg/ml) was added to exponentially growing cultures. Samples were removed, and serial dilutions were plated for counting.*

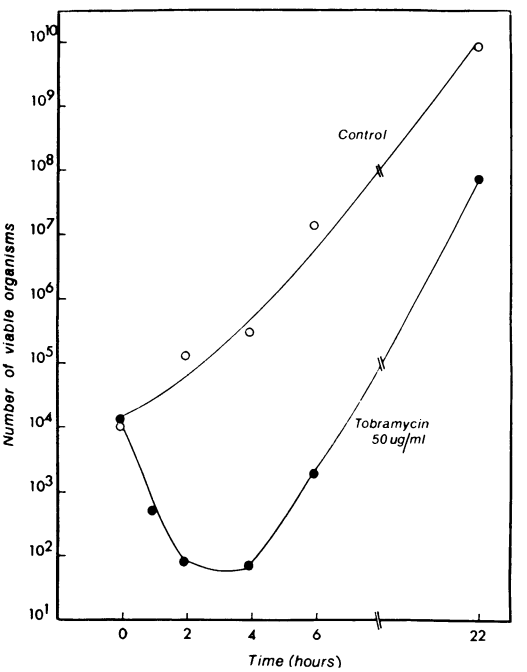


FIG. 3. *Growth of a Pseudomonas aeruginosa strain resistant to 50 μg of tobramycin/ml. Tobramycin was added to an exponential-phase culture. Samples were removed, and serial dilutions were plated for counting.*

*Klebsiella* strains examined. In view of the effect of $Ca^{++}$ and $Mg^{++}$ upon tobramycin, the EDTA effect presumably results from chelation and not from an alteration of cell wall permeability to effect entry of the antibiotic into the organism.

**Correlation of MIC and disc zones.** There was a good correlation of zone size and MIC for *S. aureus*, *E. coli*, *Enterobacter*, *Klebsiella*, *Pseudomonas*, and indole-positive *Proteus* strains. However, zones of inhibition larger than 16 mm were found for a number of *P. mirabilis*, *Providence*, and *Salmonella* strains, in spite of the fact that the MIC is 12.5 μg/ml or greater.

## DISCUSSION

Tobramycin appears to have excellent antibacterial activity against various strains of *Enterobacteriaceae*, *Pseudomonas*, and *S. aureus* collected during a period of extensive use of kanamycin and gentamicin. The average MIC values are in a range that is attainable by intramuscular

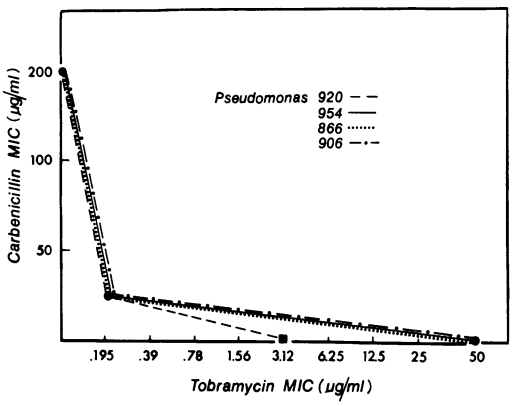


FIG. 4. *Isobologram showing combined action of tobramycin and carbenicillin against four strains of Pseudomonas aeruginosa as determined in a checkerboard broth dilution assay.*

administration (3). The fact that *Proteus mirabilis* strains were less susceptible to this agent than were other bacteria is not a serious problem, because *P. mirabilis* remains quite susceptible to ampicillin and kanamycin (Table 1). It is unfortunate that *Serratia* and *Providence* are resistant, as few agents are available to treat infections caused by these opportunists. Tobramycin is active against strains of *Pseudomonas* resistant to gentamicin. It also shows a synergistic activity with carbenicillin against *Pseudomonas* species. This in vitro synergy is similar to that already noted for carbenicillin and gentamicin. Whether this will have clinical application is unknown. The lack of activity against various streptococci and other "normal flora" can actually be regarded as beneficial.

In contrast to the findings of Wick and Wells (9), we did not find a much lower MIC when nutrient broth was used as a growth medium. The reason is unclear, unless it is the *p*H effect. It appears that tobramycin, like gentamicin, is more effective at an alkaline *p*H. The presence of magnesium and calcium, singly or in combination, interferes with the activity of tobramycin and raises the MIC. Serum did not inhibit the activity of tobramycin.

Resistant strains of *Pseudomonas* showed an initial decrease in viable-cell count, but resistant organisms persisted and grew out. Although the time course of killing appears to be first-order, the assay system does not allow this assumption because drug bound to the site of action probably continues to act after the bacteria are removed from the medium. The initial arrest of growth produced by tobramycin in resistant strains could be due to an induction phenomenon, with inactivation of the agent only at the cell surface. Further studies of resistant strains are being performed.

The exact mechanisms of resistance are not clear. Some *Serratia* and *Providence* strains do not destroy the compound but are resistant. Acetylation (2) is a possible mechanism, but phosphorylation and adenylation could also occur. The relatively high inhibitory levels against *Proteus* species are under investigation and may well be due to cell wall permeability rather than drug inactivation or ribosomal alteration. Further studies of multiply resistant organisms, particularly strains from patients who have received gentamicin or kanamycin, should be performed.

Tobramycin appears to be an effective agent that warrants clinical investigation.

ACKNOWLEDGMENTS

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